Studies on the Chemiluminescence from an O_2 and/or HOOH Adduct Derived from the Riboflavin–Copper(I) Chelate*

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ABSTRACT: This paper characterizes a biochemical redox system from which a chemiluminescence can be elicited by injection of HOOH. This system consists of an aqueous solution of riboflavin, ascorbic acid, and copper(II), $\pm O_2$, which is buffered with phosphate at a pH of 6.2. Evidence is presented which indicates the generation of the riboflavin–copper(I) chelate in the system. The riboflavin–copper(I) chelate is believed to form an O_2 "adduct" with molecular O_2 to produce the compound which can react with HOOH to give chemiluminescence, presumably *via* an O_2 and/or HOOH adduct of the riboflavin–copper(I) chelate. The riboflavin–copper(I) chelate was prepared independently, $\pm O_2$, and found to possess absorption and chemiluminescent spectra similar to those of the riboflavin–copper(II)–ascorbic acid system, $\pm O_2$. Reduced nicotinamide–adenine dinucleotide was found to substitute successfully for ascorbic acid. When glucose and glucose oxidase, as a HOOH feeder system, were "coupled" to the oxygenated riboflavin–copper(I) chelate, from either of the cited sources, a low-level chemiluminescence ("glow") was observed which showed first-order dependence upon the O_2 concentration. Flavin mononucleotide, rhodamine B, and pyronine B were found to yield chemiluminescence when substituted for riboflavin in the riboflavin–copper(II)–ascorbic acid system. Mechanisms are proposed for the generation of the riboflavin–copper(I) chelate in biochemical systems and its subsequent reaction with O_2 and/or HOOH to produce the species which yields light with excess HOOH.

In 1941, Szent-Györgyi proposed that electronic excited states might be utilized in a general way in the metabolism of living organisms, in addition to their well-known participation in bioluminescence, photosynthesis, and vision. Bioluminescence is chemiluminescence *in vivo* and when light in the visible spectrum is seen, it is evidence that electronic excited states with energies up to 80 kcal (3.5 eV) are produced. If the energy of electronic excited states is to serve an organism in a general manner, two basic criteria must be met: (1) the electronic excited states must be generated chemically in the dark, and (2) the energy generated must be utilized in some cellular function and *not lost as heat or light*.

In this respect, it was shown by Vorhaben (1965) and Vorhaben and Steele (1967) that, in at least one case, electronic excited states can be generated in a *model* nonbioluminescent biochemical redox system. This system consisted of riboflavin, ascorbic acid, cupric ions, HOOH, and phosphate buffer (pH 6.1), all in concentrations within physiological ranges. In connection with this work, Vorhaben (1965) discovered that this system without HOOH showed an increase in the elicitable chemiluminescence, with respect to the time of incubation under O_2, upon subsequent addition of HOOH. This indicated the generation of a relatively stable intermediate, X,[1] which perhaps could be isolated and characterized. The identity of X is fundamental to the question of the mechanism of the chemical generation of electronic states in this system, and it may be shown to have some functional role *in vivo*.

In a preliminary report Stone and Steele (1969) presented evidence that X was peroxidic in nature and possibly a hydroperoxide of riboflavin. In a later paper ,Stone *et al.* (1969) presented evidence for the generation of X *via* a riboflavin–copper(I) chelate intermediate which reacted with O_2 and/or HOOH to produce an O_2 and/or HOOH adduct. This adduct could react directly with HOOH to yield chemiluminescence or breakdown to give a riboflavin–hydroperoxide, which could then react with HOOH to give chemiluminescence. It is believed that the riboflavin–copper(I) chelate–O_2 adduct is the common intermediate in the photoinduced systems of riboflavin, copper(II), and HOOH (Steele, 1963; Vorhaben and Steele, 1965; Williams and Steele, 1965), and the dark induced system of riboflavin, HOOH, ascorbic acid, and copper(II) (Vorhaben and Steele, 1967), both of which yield chemiluminescence. This paper describes our recent progress toward characterizing the mechanism(s) of electronic excitation state generation in these model biochemical redox systems.

Materials and Methods

Ascorbic acid solutions were prepared, standardized, and kept as described by Vorhaben and Steele (1967). The hy-

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[1] Abbreviations used are: X, riboflavin derivative(s) generated in these studies which yield chemiluminescence in the presence of HOOH; rf·, riboflavin semiquinone; rf, riboflavin; H_2A, ascorbic acid; HA·, ascorbate semiquinone; A, dehydroascorbic acid; rf–Cu(I), riboflavin–copper(I) chelate; DABCO, 1,4-diazabicyclo[2.2.2]octane.

drogen peroxide and copper sulfate used were Baker Analyzed reagents. The riboflavin used was Roche, Type R, donated through the courtesy of Mr. P. E. Sleezer of Hoffman-La Roche. Riboflavin solutions were rendered metal free by treatment with Bio-Rad Chelex 100. Rhodamine B was obtained from Eastman and pyronine B from Allied Chemical. *p*-Dioxane was spectro quality from Matheson, Coleman & Bell, and Me_2SO was Baker's Analyzed reagent. All reagent solutions were made up in glass-distilled water treated with Chelex 100 (Bio-Rad Analytical grade, 100–200 mesh) to remove possible metal contaminants, particularly iron and copper.

Enzymes and Sources. Catalase (EC 1.11.1.6, Worthington, CTR), *Achromobacter fisheri* luciferase (Sigma, type I), and glucose oxidase (EC 1.1.3.4, Sigma type II) were the enzymes used in this study.

Absorption and emission spectra, pH, and the techniques for the elicitation and measurement of the chemiluminescences were the same as described by Steele (1963). The chemiluminescence spectra were measured as described by Vorhaben and Steele (1967). The measurement of the chemiluminescent spectrum from a low-intensity system was accomplished with the use of a Schoeffel-Thermo-Cooled Housing, Model type D 502T (Schoeffel Instrument Co., Westwood, N. J.) equipped with an RCA 1P21 photomultiplier tube. This assembly was operated at approximately −15° (the assembly permits the establishment of a temperature differential of 40° below the temperature of the coolant). The assembly was placed in the position of the photomultiplier housing of the Aminco-Bowman fluorospectrometer for the measurement of the chemiluminescent spectrum, and by using the analyzing monochromater the complete spectrum was determined by a rapid scan of the chemiluminescence elicited by HOOH injection. Ambient temperatures were 21–22°. Low-level chemiluminescences were measured using the Nuclear-Chicago No. 722 scintillation spectrometer as described by Vorhaben and Steele (1967). Kinetic studies on the decrease in absorbancy of the riboflavin–copper(I) chelate at 525 nm were followed on a Beckman DU spectrophotometer equipped with the Gilford expanded-scale recorder readout. The chart speed used was 40 in./min. A 10-mm quartz cuvet was sealed with a rubber septum held in place with black plastic electrical tape. The air was flushed out with N_2 by the use of two 2.5 in., 18 gauge hypodermic needles piercing the septum. The reaction mixture was introduced into the cuvet through one of the needles. The DU was set at an optical density range of 0.300 unit, full scale, and the HOOH was injected through the tape-masked cuvet holder and septum to initiate the reaction. Additional absorption spectra were recorded on a Perkin-Elmer double-beam, Model 4000A Spectracord.

Resolution of incubation mixtures to partially purify the chemiluminescent compound produced in the riboflavin–copper(II)–ascorbic acid–oxygen system was done on a silicic acid column. The column was prepared using Mallinckrodt CC-4 SilicAR, 100–200 mesh silica gel. It was washed with water to remove the "fines," with nitric acid to remove any metals, and then with "chelexed" water until the washings were neutral to pHydrion paper. The silicic acid was then dried and activated, overnight at 130° in a hot air oven. The solvent used for packing the column with silicic acid and the resolution of the incubation system was benzene–methanol (80:20, v/v). The riboflavin–copper(II)–ascorbic acid–O_2 system (referred to hereafter as the standard incubation system) was reduced to near dryness under vacuum, extracted with methanol, and then taken to near dryness again. This was repeated several times to remove all water. The final methanol "extract" was allowed to stand until the suspension of phosphate salts and other materials had settled. The clear yellowish-orange supernatant was removed then for resolution on the column. Immediately before application of the extract to the column, it was diluted with benzene to a benzene–methanol ratio of 80:20. Ten milliliters of this diluted extract was then placed on the column and resolved with the benzene–methanol column solvent with an effluent flow rate of 100 ml every 15 min (5 ml/45 sec). Five-milliliter fractions were collected and assayed on the scintillation spectrometer for elicitable chemiluminescent potential by extraction with 5 ml of 0.04 M PO_4 buffer, followed by injection of 0.1 ml of 30% HOOH as described by Vorhaben and Steele (1967). Samples of 10 μl were assayed for riboflavin, lumiflavin, lumichrome, etc., using thin-layer chromatography. Standards were prepared using Roche type R riboflavin, lumichrome from Aldrich, and lumiflavin synthesized by Dr. Krumdieck at the University of Alabama.

Thin-layer chromatography was performed on the various incubation mixtures used in this study. Silica gel plates were prepared on 8 × 8 in. glass plates using Kensco silica gel G (E. Merck AG-Darmstadt 7731, lot F203) or Kensco silica gel HF-254 + 366 (E. Merck AG-Darmstadt 7741, lot F1019). These plates were used for preparative work. For the general screening of mixtures for the determination of reaction mixture components before and after incubation or chemical treatment, 5 × 20 cm precoated plates (Quanta-Gram Q1 and Q1-F, Quantum Industries, Fairfield, N. J.) and 20 × 20 cm precoated sheets (Baker-Flex silica gel 1B and 1B-F, Baker Chemical Co.) were used. The Quanta Gram plates were used as shipped. The Baker-Flex sheets were cut to the desired size, activated at 120° for 3 hr in a hot air oven, and stored in a desiccator until used. The solvent used for thin-layer chromatography in all but one instance was the same as used for the column chromatography. The exception was the solvent used for the determination of ring rupture occurring in riboflavin during the incubation under O_2. The resulting amines were visualized with ninhydrin, and the solvent used was 1-butanol–glacial acetic acid–water (4:1:5, v/v, as described by Mager and Berends, 1965).

The hydroxylation of aromatic substrates was determined on thin-layer chromatograms using Gibb's reagent (*N*,2,6-trichloro-*p*-benzoquinone imine, 0.5% in absolute ethanol, w/v) as described by Randerath (1964). A developed thin-layer chromatography plate could be sprayed with Gibb's reagent, dried, and then sprayed with 10% sodium carbonate solution (aqueous, w/v). The hydroxylated aromatic compounds on the plate displayed as blue, green, brown, or gray spots, depending on the compound and its concentration. Standard compounds used were phenol, quinhydrone, salicylic acid, benzoic acid, and gentisic acid. α-Benzoin oxime (5% in absolute ethanol, w/v) was used as described by Feigl (1946) for the visualization of copper-containing spots on thin-layer chromatography. A positive test gave a green, chartreuse, or blue spot, depending on the concentration of the copper and its form. Phosphate was visualized on thin-layer chromatography as a blue spot when the plate was sprayed with

aqueous 1% ammonium molybdate (w/v) and 1% stannous chloride (w/v) in 10% hydrochloric acid (Stahl, 1965).

Tests for peroxides in solution were made with the reagent described by Snell and Snell (1949), consisting of ferrous ammonium sulfate and thiocyanate. The solution was modified to the extent that ammonium thiocyanate was used mole for mole in place of potassium thiocyanate. This reagent was used to test for all peroxides in solution. Hydrogen peroxide caused the formation of the red ferric thiocyanate complex, but in the presence of riboflavin, this color was changed to a yellowish-orange. Ferrous ion in the presence of thiocyanate iron and riboflavin gave a turbid white color. All peroxide tests were conducted anaerobically to eliminate the possibility of air oxidation of the reagent to give a false positive test.

All procedures for preparing, performing, or assaying incubation mixtures, and all procedures involving any oxygenated riboflavin–copper chelate, were carried out either in the dark or under red light. Low actinic glassware or shielded apparatus, including chromatography columns and tanks, were used. This was necessary to avoid light sensitization of labilization of the chemiluminescent compound(s), their precursors, or derivatives.

Two major systems for the generation of the chemiluminescent compound were used: (1) the standard incubation system consisting of an aqueous PO_4-buffered solution of dye (*e.g.*, riboflavin), copper(II), ascorbic acid, and molecular oxygen (present during or added after incubation); and (2) an aqueous solution of dye, acetonitrile–copper–complex salt ($Cu^+(CH_3CN)_4ClO_4^-$), and molecular oxygen (added after incubation). The complex salt, $Cu^+(CH_3CH)_4ClO_4^-$, was synthesized using a modification of the method given by Hemmerich and Sigwart (1963).

A cross "calibration" for light detection was established between the 1P28 photomultiplier instrumentation and the scintillation spectrometer with the precautions noted by Vorhaben and Steele (1967). On the average, one minor scale division on the Honeywell strip-chart paper used (9284N) was designated as 0.1 relative chemiluminescent unit with the ammeter set to read 3×10^{-9} A full scale (equal 10 relative chemiluminescent units), and was equal to 2×10^5 apparent counts on the scintillation spectrometer.

Results

The following parameters, as noted, were examined for their affect upon the standard incubation system (Methods).

Dye Specificity. The dyes rhodamine B, pyronine B, and riboflavin were examined under the same incubation conditions for their relative ability to develop an elicitable chemiluminescence potential which displays upon the injection of HOOH. Representative responses elicited from these systems after 2-hr incubation at ambient pO_2 are presented in Figure 1. For comparing the relative responses for different dyes or for one dye in relation to a given parameter only the chemiluminescent intensities at the maxima of the curves, as shown in Figure 1, were plotted. The peak intensity of the chemiluminescence elicited from rhodamine B was greater than for either the riboflavin or pyronine B incubated for the same length of time, but the total chemiluminescence was greatest for riboflavin. Pyronine B showed much less potential for either peak chemiluminescent intensity or total light emitted.

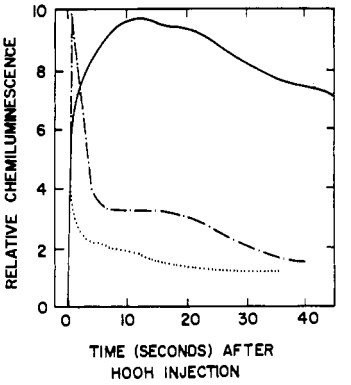

FIGURE 1: Chemiluminescence, elicited by the injection of 5 mmoles of HOOH, *vs.* time from copper–ascorbic acid dye systems after 2-hr incubation at 21° at ambient pO_2. Systems: riboflavin (—), 0.87 μmole; ascorbic acid, 7.1 μmoles. Rhodamine B (— · —) and pyronine B (· · · ·): 0.45 μmole of dye and 8.2 μmoles of ascorbic acid. All systems contained 3 μmoles of Cu(II) and 20 mmoles of PO_4 buffer (pH 6.1); final volume, 15 ml.

pO_2. When the standard incubation system with riboflavin was incubated under N_2, a nominal chemiluminescent potential was observed to build up within 10 min. This potential was found to be constant for incubation periods up to 26 hr. However, when pure O_2 was bubbled through the incubation mixture, a very rapid increase in the chemiluminescent potential was observed. This potential not only increased very rapidly, it yielded much more chemiluminescence, by an order of magnitude, than a comparable system incubated under a lower pO_2 (*cf.* Stone *et al.*, 1969, Figure 1). Since the chemiluminescence potential of a standard incubation system was enhanced so greatly by an increase in O_2 tension, the use of pure O_2 was instituted routinely in order to obtain the greatest concentration of the chemiluminescent compound in the least time for all other experiments involving the oxygenated system.

pH. The effect of pH upon the generation of the chemiluminescent compound, and its ultimate chemiluminescent elicitation, was determined. When systems were *both* incubated *and* elicited at pH's ranging from 2 to 9, the greatest chemiluminescence was obtained between pH 5.8 and 6.4. If the incubation systems prepared at various pH's were all adjusted to pH 6 prior to HOOH injection, the greatest chemiluminescence was obtained at an incubation pH of 7. It was observed that very rapid bleaching of the riboflavin yellow color occurred upon HOOH injection if the pH was greater than 6.5; consequently, a pH of 6.2 was considered optimal for both the generation of the chemiluminescent compound and the elicitation of chemiluminescence therefrom.

Light. Illumination of a standard incubation system with unfiltered light from a 500-W TDC (Three Dimension Co., Division of Bell and Howell) projector had no effect upon the chemiluminescence elicited subsequently from the system as long as oxygen was excluded. Molecular oxygen reacted with the components of the incubation system to cause an increase in chemiluminescent potential with time of incubation. This chemiluminescent potential was reduced markedly if the system were illuminated with light subsequent to incubation.

Metals. If an incubation system was treated with Chelex 100 ion-exchange resin *either* before or after incubation,

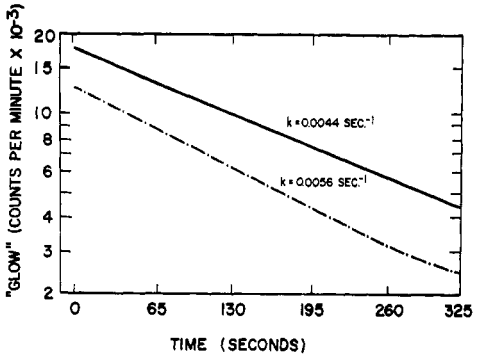

FIGURE 2: Semilog plots of chemiluminescent glow *vs.* time in a glucose oxidase–HOOH feeder system. System incubated under O_2 for 5.5 hr, and the glow elicited by the injection of glucose + glucose oxidase. System riboflavin, 7 μmoles; ascorbic acid, 14 μmoles; Cu(II), 1 μmole; PO_4 buffer, pH 6.1, 15 mmoles; glucose, 178 mmoles; glucose oxidase, 435 units. Volume, 14.5 ml; temperature, 21°. (——) Counts elicited immediately following the injection of the HOOH feeder system. (·—·—·) Counts following O_2 gassing subsequent to the injection of the HOOH feeder system.

more than 90% of the chemiluminescent potential was lost. Since Chelex 100 is an effective chelator of copper and iron ions it was reasonable to assume that metals (most probably the added copper(II) ion) are involved directly in the generation, or structure of the chemiluminescent compound.

HOOH. Vorhaben and Steele (1967) found in studies with the standard incubation system a very rapid buildup upon oxygenation of an elicitable chemiluminescent potential. This same system, after about 5-min incubation, displayed also an *unelicited* low level chemiluminescent "glow," *i.e.*, an *autochemiluminescence*. It is known that HOOH is generated in an aqueous solution of ascorbic acid, copper(II), and O_2 (Lyman *et al.*, 1937; Hand and Greisen, 1942; Khan and Martell, 1967). We propose that the HOOH generated in this system reacts with the chemiluminescent compound generated in the incubation to yield the glow. This proposal is supported by the fact that the addition of powdered manganese dioxide very effectively quenches the glow (also compare results to catalase below). In addition, the presence of MnO_2 *during* the aerobic incubation decreased the glow while increasing the elicitable chemiluminescent potential with respect to the time of incubation. It seems likely that the manganese dioxide acts by destroying the endogenously generated HOOH (Gomez and Riva, 1948). This prevents the reaction between the endogenous HOOH and the chemiluminescent compound and allows the latter to accumulate more rapidly. If very small amounts of HOOH are introduced into the oxygenated MnO_2-containing system subsequent to incubation a low level chemiluminescence is seen identical with the glow produced in an oxygenated system without MnO_2. The addition of MnO_2 to an incubation system subsequent to incubation has no impact on the elicitable chemiluminescence, and was used as internal control in experiments with MnO_2 present during the incubation.

Solvent. Changing the nature of the solvent by the addition of *p*-dioxane, Me_2SO, or 1,4-diazabicyclo[2.2.2]octane (DABCO, Aldrich Chemical Co.) had marked effects upon the elicitable chemiluminescence depending on when the substance was added. In general, if the material was present during the incubation, the chemiluminescent potential was greatly decreased; if the material was added subsequent to incubation and before HOOH injection, chemiluminescence was greatly enhanced. When, *e.g.*, the standard incubation system was incubated (4 hr) with *p*-dioxane present (1.5 ml in a total 15-ml volume) only about half the elicitable light was obtained as from a system containing no *p*-dioxane. When, however, the *p*-dioxane was added to the system subsequent to incubation and before the addition of HOOH about three times as much total light was elicitable as from the standard incubation system. When *p*-dioxane was added to a glowing system, the glow was greatly enhanced. Even though no difference was noted in the action between cleaned and untreated dioxane, the *p*-dioxane was treated with activated alumina (Dasler and Bauer, 1946) to remove peroxides before it was used in these experiments. DABCO is reported to be an effective and efficient quencher of excited singlet oxygen (Ouannes and Wilson, 1968). It was used in an experiment similar to that used for dioxane and Me_2SO to ascertain if their enhancement of chemiluminescence was due to excited singlet O_2 ($^1\Sigma_g^+$) enhancement by lowering the polarity of the solvent, since increased solvent polarity quenches this oxygen species (Arnold *et al.*, 1965). The addition of a few crystals of DABCO to any standard incubation system caused a marked enhancement of the chemiluminescence. This enhancement was not quantified with respect to the amount of DABCO used but did appear to be directly proportional.

Enzymes. The enzymes catalase, glucose oxidase, and *Achromobacter fischeri* luciferase were studied for their effect on the elicitable chemiluminescence. The presence of catalase in an incubation system during the incubation did not prevent the chemiluminescent compound from accumulating though it did reduce the glow (see MnO_2 results, above) and the total light elicitable by HOOH injection. The latter reduction in intensity, however, may be due to the rapid destruction of added reagent peroxide by the catalase, decreasing thereby its effective concentration compared with the control system containing no enzyme. Further, the addition of catalase to a postincubation system had no influence on the light emitted by subsequent HOOH addition. This result indicated that the chemiluminescent compound is not sensitive to destruction by catalase.

Glucose oxidase oxidizes glucose to gluconic acid with the simultaneous production of HOOH. We proposed (above) that the glow seen in the incubation systems was the result of the chemiluminescent compound reacting with endogenously generated HOOH. Consequently glucose oxidase plus glucose and O_2 should generate sufficient HOOH to increase the glow in an incubating system. Furthermore, the glucose oxidase and glucose concentrations can be adjusted to generate HOOH at a relatively constant rate at low concentrations. This feeder system for the generation of HOOH could then be coupled with the chemiluminescent compound to yield a parameter measurable in extremely small quantity, namely individual photons generated by the reaction of the chemiluminescent compound with HOOH. The effect of this HOOH feeder system on an oxygenated incubation system is seen in Figure 2. This reaction may be useful as a prototype model system for enzyme assay for reactions yielding HOOH as a product. Parenthetically, recent experiments indicate that this assay may be made two to three orders of magnitude more sensitive by counting the light in the mono- or summation mode on the

scintillation spectrometer rather than in the coincidence mode (see, *e.g.*, Vorhaben and Steele, 1967).

The reduced form of flavin mononucleotide ($FMNH_2$) has been cited by Strehler *et al.* (1954) as likely being bacterial luciferin. Hastings *et al.* (1966) have presented data to indicate that the luciferin reacts with O_2 to produce an intermediate which reacts then with aldehyde to yield light. Since Vorhaben (1965) and Stone and Steele (1969) have shown that FMN can be substituted for riboflavin in the generation of the chemiluminescent compound it was of interest to determine whether the compound would react with the bacterial luciferase to give light. When the chemiluminescent compound generated in either a riboflavin or FMN incubation system was oxygenated, reacted with dodecylaldehyde (Aldrich Chemical Co.), solubilized by sonication, and then injected with the luciferase, no chemiluminescence was elicited. Changing the order of addition of the reagents was without effect.

Thin-Layer Chromatography Data. A positive peroxide test was obtained with an oxygenated incubation system (flushed with N_2 before testing) but not with an unoxygenated system. When the incubation system was resolved on silica gel thin-layer chromatography, two copper-containing spots were found. These two spots had R_F values of 0.00 and 0.05 (solvent benzene–methanol, 80:20, v/v) and both were active for chemiluminescence. No other areas of the thin-layer chromatographic plate were chemiluminescent active. Control experiments showed that copper alone was not responsible for the observed chemiluminescence. The copper-containing spot at the origin of the thin-layer chromatographic plate was present in all incubation systems, but the copper-containing spot at R_F 0.05 was found only in oxygenated systems. We suggest that this new spot is an oxygen adduct of the chemiluminescent compound and is peroxidic in nature; indeed it may be the hydroperoxide of riboflavin at position C-4a as suggested by Hemmerich (1968).

In the generation of the chemiluminescent compound in an incubation system it appears that two intermediate compounds have been identified. Cuprous ion is involved in an early generation step and either a HOOH and/or O_2 adduct is generated later. When HOOH is substituted for O_2 in an incubation mixture, the chemiluminescent compound is seen to increase with respect to the time of incubation, but it accumulates more slowly and in smaller amount. Evidently, in the presence of excess HOOH, the chemiluminescent compound is formed by reaction with HOOH directly, or directly with O_2 produced endogenously from HOOH plus Cu(II), or a combination of these. We consider the failure of the chemiluminescent compound to accumulate in the presence of HOOH to be due both to insufficient oxygen and increased destruction of the compound by the exogenously added HOOH since the latter is known to enhance the glow from the incubation system.

Dye Requirement. In experiments designed to ascertain the function of the dye in the incubation systems, incubations were carried out without the riboflavin present. The dye was added subsequent to incubation and immediately before the elicitation of the chemiluminescence by the addition of HOOH. In a representative system, an incubation mixture was prepared which contained per 14.5-ml aliquot: PO_4 buffer, pH 6.2, 4 mmoles; Cu(II), 3 μmoles; H_2A, 8 μmoles. This mixture was incubated under O_2 for various periods of time and duplicate aliquots were removed, one injected with

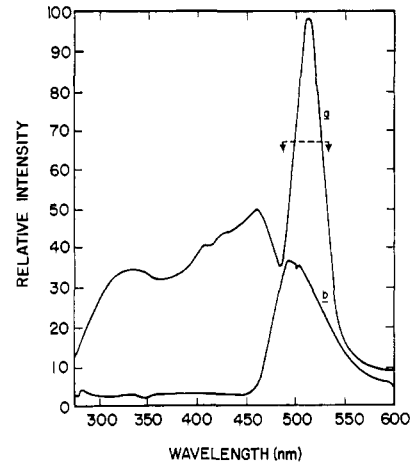

FIGURE 3: Fluorescent spectrum (curve b) and the activation spectrum (curve a) of a silicic acid column cut (see text) containing the compound from which chemiluminescence can be elicited by the addition of HOOH (see Figure 9). Emission monochromator set at 510 nm for the activation spectrum; activation monochromator set at 325 nm for the fluorescent spectrum. – – – indicates scattered exciting light.

0.52 μmole of riboflavin and the other injected with an equal volume of water as a volume control. These aliquots were then assayed immediately by the injections of 5 mmoles of HOOH and the chemiluminescence was recorded with the 1P28 instrumentation (Methods). The chemiluminescence observed at zero time (6-min incubation) was slight and became less as the incubation time increased. If the dye were functioning solely as an antenna (designated as fluorescers by some authors) for the emission of electronic excitation states generated elsewhere in the system, the chemiluminescent intensities elicited in these experiments should have been the same as those elicited from systems incubated in the presence of the dye. The nominal emission elicited under these conditions is attributable to the known reaction of HOOH with copper(II) to produce light; this can be enhanced markedly by the presence of an antenna (R. H. Steele, unpublished data; Vorhaben and Steele, 1967, Figure 2, curve d).

The results seem conclusive that the riboflavin is more than just an antenna. There is good reason to believe that the chemiluminescent compound is a riboflavin derivative but further discussion will be delayed until additional data have been presented.

Spectra. The methanol extract of an incubation system, mentioned above, was purified partially on a silicic acid column (benzene–methanol, 80:20, v/v). The fluorescent and activation spectra (curves b and a, respectively) of the partially purified X compound (chemiluminescent compound) are presented in Figure 3. The fluorescent emission (495 nm) is blue shifted with respect to riboflavin which exhibits a λ_{max} of 520 nm (uncor). The chemiluminescent spectrum was recorded by eliciting the chemiluminescence in the Aminco Bowman fluorimeter (no exciting light), and scanning the emission with the analyzing monochromater. A cooled 1P21 photocell (Methods) was used to detect the light with the response read on an X-Y recorder. This spectrum is presented in Figure 4. The chemiluminescent spectrum extends from about 400 to 600 nm with the maximum at about 520 nm,

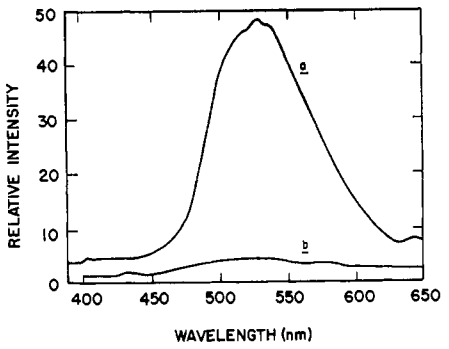

FIGURE 4: Chemiluminescent spectra from a partially purified (silicic acid column cut; see text) sample from a standard incubation system (curve a), and from a highly purified sample (curve b). The spectrum of a was recorded using a cooled RCA 1P21-photomultiplier tube (Methods); b was measured at ambient temperature.

which coincides with the fluorescence of riboflavin and is similar to the chemiluminescent spectrum obtained from a similar system by Strehler and Shoup (1953) and Vorhaben and Steele (1967).

Glow. The data plotted in Figure 5 show the glow and elicitable chemiluminescence potential (curve maxima are recorded as described in the text for Figure 1) of a standard incubation system at various incubation times. In every case, the glow in an incubation system peaks long before the chemiluminescence potential does. The fact that HOOH is generated in the incubation system implies that the glow might be a direct consequence of the HOOH production and therefore the glow would diminish as the HOOH generation diminished. Vorhaben (1965) reported that all the H_2A was exhausted in less than 1 min in such systems, but it is possible that the ascorbate oxidation products or the ascorbate semiquinone–copper–oxygen complex proposed by Khan and Martell (1967) have much longer lifetimes. As reported by us in an earlier paper (Stone *et al.*, 1969), the pseudo-first-order rate constants for the chemiluminescent decay elicited from an incubation system are relatively unchanged over a considerable portion of the incubation period. It is consequently

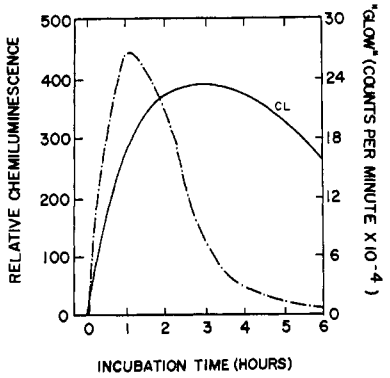

FIGURE 5: Maximum chemiluminescence and glow *vs.* incubation time from a system containing riboflavin (0.46 μmole), ascorbic acid (8 μmoles), Cu(II) (3 μmoles), PO_4 buffer (pH 6.2, 4 mmoles), under 100% O_2 at 1 atm. The chemiluminescence was elicited by the injection of 5 mmoles of HOOH. The glow is autochemiluminescence. Final volume, 15 ml; temperature, 21°.

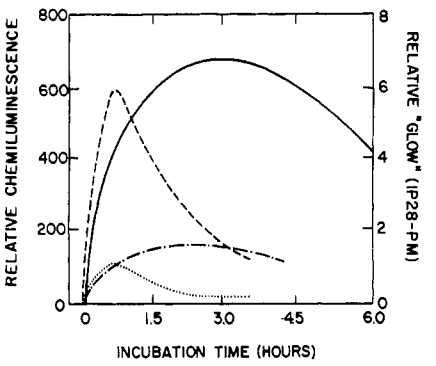

FIGURE 6: Maximum elicitable chemiluminescence and glow *vs.* incubation time of a standard system containing sodium benzoate as an hydroxylatable substrate. System: riboflavin, 7 μmoles; ascorbic acid, 21 μmoles; Cu(II), 1.45 μmoles; PO_4 buffer (pH 6.0), 4 mmoles; benzoate when used, 100 μmoles; chemiluminescence elicited by the injection of 5 mmoles of HOOH. (—) No benzoate in the system; (—·—) benzoate present; (----) glow in the absence of benzoate; (····) glow in the presence of benzoate. Final volume, 15 ml.

unlikely that more than a single chemiluminescent species is present and yielding chemiluminescence in the reaction with HOOH.

Hydroxylation. Williams and Steele (1965) reported that aromatic hydroxylation takes place during the photoinduced chemiluminescence of riboflavin in an aqueous solution of HOOH, with benzene and benzoic acid as substrates. This reaction was catalyzed markedly by copper ions. These substrates, which were found to inhibit the chemiluminescence, were believed to act by trapping hydroxyl radicals. This belief was supported by the demonstration of phenol and salicylic acid generation in systems containing benzene and benzoic acid, respectively. Vorhaben (1965) found similar results when benzoic acid was incorporated in the dark induced standard incubation system, namely, the inhibition of chemiluminescence and the formation of salicylic acid. Gentisic acid was also produced in this system. We present data in Figure 6 showing the results of incubating sodium benzoate with the standard incubation system. Aliquots of the various systems were taken before and after all reaction steps to detect the formation of any hydroxylated compounds. The thin-layer chromatography study showed the formation of gentisate in an incubating system and in *all* aliquots to which HOOH had been added. A control experiment showed that gentisate could be formed from benzoate in water containing only Cu(II), O_2, and HOOH. Therefore, the formation of gentisate in aliquots *subsequent* to chemiluminescent elicitation is not significant of itself and is probably due to the hydroxylation mechanism proposed by Konecny (1954). The knowledge that HOOH is generated early in the incubation of a standard system under O_2, and in the presence of Cu(II), may account for most of the hydroxylation that occurs. From this, it can be reasoned that the hydroxyl radicals formed are *not functional per se* in the generation of the chemiluminescent compound X (see also remarks below). The inhibition of the chemiluminescence might be simply a result of competition for the Cu(II) between the ascorbate semiquinone and the hydroxylated substrate *via* the directed *o*-hydroxylation mechanism proposed by Brackman and

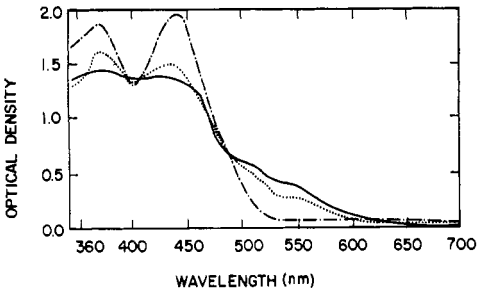

FIGURE 7: Absorption spectra (10-mm light path) of an artificial incubation system prepared *in vacuo* (——), and exposed successively to O_2 (· · · · → —·—). Initial concentrations for the formation of the riboflavin–Cu(I) chelate in a 5-ml volume: riboflavin, 0.7 μmole; ascorbic acid, 4 μmoles; Cu(II), 0.7 μmole; PO_4 buffer, pH 6.2, 0.2 mmole. Temperature 21°.

Havinga (1955). This would account for the fact that the hydroxylatable substrate exerts its maximum effect *early* in the incubation, when proposed formation of the ascorbate semiquinone–Cu(I) complex occurs, leading to the generation of the rf–Cu(I) chelate.

Riboflavin–Cu(I) Chelate. As expected, the maximum chemiluminescent potential of an incubation system is reached much more rapidly and more light is produced when the incubation mixture is oxygenated *after* the riboflavin, ascorbate, and Cu(II) are incubated anaerobically, since there is no competition between copper and oxygen for the ascorbate. The riboflavin, ascorbate, and Cu(II) were incubated together under N_2 in phosphate buffer (pH 6.2) for 20 min. A dark red solution was obtained with the color changing slowly from the riboflavin yellow to red within 5 min. The absorption spectrum of this system is presented in Figure 7 and is quite similar to the spectrum of the rf–Cu(I) chelate (Stone *et al.*, 1969). When the system was oxygenated, the red color faded and the riboflavin yellow color reappeared (Figure 7). The chemiluminescent potential of this system increased by an order of magnitude after 10-min oxygenation. Analogous results were obtained when reduced nicotinamide–adenine dinucleotide (NADH) was substituted for ascorbate mole for mole.

A Bjerrum (1941) titration was carried out on the anaerobic incubation system with ascorbate and gave the same results as described by Hemmerich and Spence (1966). The system turned red, during the titration, at a pH of approximately 3.8. It is evident that the rf–Cu(I) chelate was generated and is responsible for the formation of the red color and the titratable hydrogen. Consequently, the rf–Cu(I) chelate may also be postulated as being generated in the standard incubation system.

The marked similarity of the incubation systems, anaerobic and oxygenated, with respect to the effect of oxygenation, thin-layer chromatography findings, chemiluminescent elicitation, and decay kinetics (Stone *et al.*, 1969) indicates strongly that the rf–Cu(I) chelate is a common intermediate in all the systems studied.

Discussion

In the course of this study, a number of chemical and physical properties of the incubation system were determined

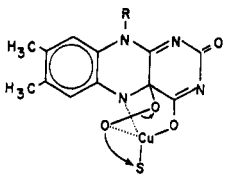

FIGURE 8: The riboflavin–Cu(I)–O_2 adduct–substrate complex proposed by Hemmerich (1968) to be involved in the enzymatic oxidation of substrate. S represents the substrate to be oxidized.

which implied generally that the X (chemiluminescent compound) was a riboflavin derivative, probably a peroxide and possibly containing paper. Vorhaben (1965) had postulated the formation of a riboflavin hydroperoxide, which Hemmerich (1968) postulated to be the C-4a derivative in his systems. Two other possibilities have been proposed for riboflavin peroxides: a rf–OOH at C-1a (Mager and Berends, 1965), and an endoperoxide between C-1 and C-4 in riboflavin (Reid, 1957).

The chemiluminescence elicited from all systems studied, $\pm O_2$, displayed pseudo-first-order kinetics and the rate constants in each case were essentially the same or similar (Stone *et al.*, 1969). The rf–Cu(I) chelate had been proposed by Vorhaben and Steele (1965) as a possible intermediate in the mechanism by which copper ion enhanced the chemiluminescence in the photoinduced riboflavin–HOOH system. The addition of O_2 to the rf–Cu(I) chelate, to form the quasi-O_2 adduct (Figure 8, Hemmerich, 1968), would reduce the polarity of the compound if the directed bond from O_2 to copper is considered to be covalent in nature. This would effectively cancel the charge on the copper. This corresponds with the appearance of the new spot, in the oxygenated system, on thin-layer chromatography using benzene–methanol (80:20, v/v) with an R_F of 0.03–0.05. The formation of the riboflavin–OOH (at C-4a) would also decrease the polarity of the compound. However, the thin-layer chromatography data indicate the presence of copper in the chemiluminescent-active compounds so it appears likely that the O_2 adduct is a derivative of the rf–Cu(I) chelate.

The reaction of the rf–Cu(I) chelate with O_2 and/or HOOH is postulated by us to proceed *via*

$$\text{rf–Cu(I)} + O_2 \longrightarrow \text{rf–Cu(I)–}O_2 \qquad (1)$$

which can react with HOOH

$$\text{rf–Cu(I)–}O_2 + \text{HOOH} \xrightarrow{H^+} \text{rf–OH} + \text{Cu(II)} + O_2 + H_2O + \text{light} \qquad (2)$$

We present in Figure 9 a spectral summary for the riboflavin system(s)' special characteristics as an aid in evaluating the energetics of the chemiluminescent data which are the subject of this paper. The large amount of energy required for the chemiluminescence displayed in the emission spectrum (Figures 4 and 9) of at least 75 kcal (3.25 eV) is greater than can be accounted for by the riboflavin fluorescence which has its 0–0 transition at approximately 495 nm (58 kcal, eV). The presence of the fluorescent species in the incubation mixture (curve b′, Figure 9) whose emission is blue shifted with respect to riboflavin is probably a riboflavin derivative (rf–OH of eq

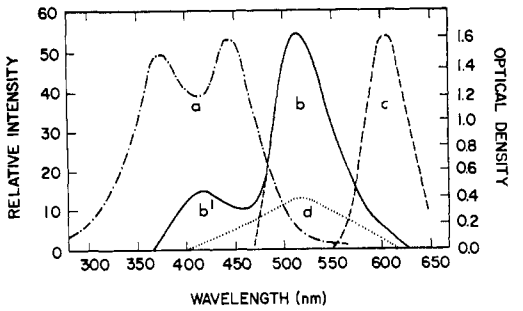

FIGURE 9: Spectral data for riboflavin: curves a, b, and c are the absorption, fluorescent, and phosphorescent (Steele, 1963) spectra, respectively; curve b',b is the fluorescent emission on a standard incubation system (see text) *subsequent to the elicitation* of chemiluminescence (curve d) by HOOH addition.

2 and 7) and may account for the broad chemiluminescent spectrum of curve d, Figure 9.

When HOOH reacts directly with the rf–Cu(I) chelate formed anaerobically, the chemiluminescence elicited is less intense than the chemiluminescence obtained from the oxygenated chelate, and the decays are different kinetically (Stone *et al.*, 1969). It was noted also that a flash (rapid buildup) of chemiluminescence was obtained when HOOH was added to the oxygenated chelate system in contrast to the slower buildup in the emission intensity when the HOOH was added to an unoxygenated chelate system. The reactions of the unoxygenated chelate plus HOOH system may proceed as follows

$$\text{rf–Cu(I)} + \text{HOOH} \longrightarrow \text{rf–Cu(I)–OOH} + \text{H}^+ \qquad (3)$$

followed by the reaction of the HOOH adduct with the excess HOOH as

$$\text{rf–Cu(I)–OOH} + \text{HOOH} \longrightarrow \text{rf–Cu(I)–OH} + \text{H}_2\text{O} + \text{O}_2 \quad (4)$$

or more probably

$$\text{rf–Cu(I)–OOH} + 2\text{HOOH} \longrightarrow \text{rf} + \text{Cu(II)} + 2\text{H}_2\text{O} + \text{O}_2 + \cdot\text{OH} + \text{OH}^- \quad (5)$$

The exothermicity of this reaction is sufficient to produce O_2 in an excited singlet state which could then react with fluorescent riboflavin to produce light *via* the Khan–Kasha (1966) mechanism.

The possibility of the formation of a double O_2–HOOH adduct of the rf–Cu(I)–chelate should also be considered, such as

$$\text{rf–Cu(I)–O}_2 + \text{HOOH} \rightarrow \text{rf–Cu(I)(OOH)–O}_2 + \text{H}^+ \qquad (6)$$

which would have the structure obtained by substituting HOO^- for S in Figure 8. It is probable that such a double adduct would be unstable and exist only as a short-lived intermediate which would react with excess HOOH in a concerted mechanism as

$$\text{rf–Cu(I)(OOH)–O}_2 + 2\text{HOOH} \longrightarrow \text{rf–OH} + \text{H}_2\text{O} + \cdot\text{OH} + \text{OH}^- + 2\text{O}_2 \quad (7)$$

with the riboflavin hydroxylated at position C-4a by analogy with the mechanism proposed by Viscontini and Okada (1967). Parenthetically, we have written free-radical species in reactions 5 and 7 to balance the equations and to account for the free-radical reactions known to proceed in copper–HOOH systems (Baxendale, 1952).

The possibility that the riboflavin complex is an adduct formed *via* a peroxide radical with riboflavin cannot be ruled out unambiguously (Stone *et al.*, 1969) in spite of the thin-layer chromatography data which indicates that the complex contains copper. For this reason the following mechanism as proposed by Hemmerich (1968) may be possible

$$\text{rf–Cu(I)–O}_2 + \text{H}^+ \longrightarrow \text{rf–OOH} + \text{Cu(II)} \qquad (8)$$

Allusion should be made to the peroxidic structure proposed for the riboflavin–Cu(I)–chelate (Figure 8) and its resemblance to the dioxetane peroxide ring system which McCapra (1968) emphasizes may undergo a concerted decomposition to yield products containing a carbonyl function in an electronically excited state. The redistribution of this energy within a fluorescent molecular species may then lead to light emission. This McCapra-type chemiluminescence (McCapra and Richardson, 1964; McCapra and Chang, 1966; McCapra, 1968; McCapra *et al.*, 1968; however, see also Rauhut *et al.*, 1965) appears to be emerging as a common mechanism for many chemiluminescent reactions, particularly in the realm of bioluminescence as emphasized by Hastings and Morin (1969). We recognize that an analogous mechanism may occur in our system with ring rupture between C-4 and C-4a to give an excited carbonyl function at position C-4a. In the oxygenated incubation systems it is probable that the overall effect is not a single series of reactions but rather a combination of the proposed reactions in competition with each other for the various intermediates.

The enhancement of the chemiluminescence by dioxane, Me_2SO, and DABCO may be the result of a weakening of the chelate structure by interference with the O → Cu or N → Cu directed bonds by the unshared electron pairs of these species.

If the riboflavin complex proves isolable and stable it may prove useful for the assay of enzymes involved directly or indirectly in the production or destruction of HOOH by measuring the HOOH with the light reaction.

In addition to the possibility that the FMN–Cu(I) chelate quasi-O_2–HOOH adduct may be bacterial luciferin or a model thereof, there is a possible connection with the microsomal oxidation systems in which ascorbic acid, reduced nucleotides, and flavins are involved (Schneider and Staudinger, 1964). Further, microsomes are known to contain considerable copper (Mason *et al.*, 1965), the function of which is not yet apparent. The fact that NADH can substitute for ascorbic acid in the formation of the copper chelate from riboflavin or flavine mononucleotide makes feasible the idea that a flavin–Cu(I) oxygen adduct may function in biological systems. This system may react in concert with cytochrome P-450 to yield the activated oxygen functional in biological hydroxylations. This thesis certainly merits attention.

We submit that this work emphasizes the versatile functional roles of riboflavin and its derivatives in biological redox energetics. Where it was thought initially to be primarily an electron transmitter involved somehow in oxidative phosphorylation it now appears that it may be involved also in

oxygen activation and in the generation of electronic excitation states.

Certainly new prospects for the participation of chelates involving different metals and ligands in biochemical redox energetics and oxygen activation are suggested by this work. The activation by Ca^{2+} of the photoproteins from different bioluminescent species (Shimomura and Johnson, 1966; Hastings *et al.*, 1969), and the influence of Zn^{2+} and Ca^{2+} on the spectrial emission from the firefly system (Seliger and McElroy, 1966), probably indicates chelation involvement in the energetics of these reactions. Whether or not these highly energetic reactions may be found to have a functional role, particularly in man, is, of course, a moot point. All of these reactions appear to involve an irreversible destruction of the participating molecules and such losses, particularly where that molecule may be a vitamin, would tend to preclude a functional role for the process unless a mechanism emerges which preserves the structural integrity of the activated species.

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